REMARKS

This application has been carefully reviewed in light of the final Office Action dated July 19, 2006. Claims 192, 194, and 195 are in the application, of which Claim 192 is independent. Claims 198 to 201 have been cancelled without prejudice to Applicants' right to present these claims in a later-filed divisional application. Reconsideration and further examination are respectfully requested.

Claims 192, 194, and 195 were rejected under 35 U.S.C. § 101 as not being supported by either a specific or substantial asserted utility or a well-established utility. In a related rejection, Claims 192, 194, and 195 were rejected under 35 U.S.C. § 112, first paragraph, on the grounds that one skilled in the art would no know how to use the invention. These rejections are respectfully traversed, for at least the following reasons.

A. The specification sets forth a specific and substantial asserted utility.

Applicants asserted, at page 178, lines 27 to 32 of the specification, that clone bn97_1 shares activity with oxidized LDL (designated LOX-1). In particular, Applicants asserted that the bn97_1 protein would share an activity of binding oxidized low-density lipoproteins, internalizing them into endothelial cells, and destroying them, and thus would play a crucial role in the pathogenesis of atherosclerosis.

Applicants respectfully submit that this is an assertion of a specific and substantial utility, i.e., a utility which is specific to the claimed subject matter and which describes a "real world" use. See MPEP § 2107.01(I)(A) and (I)(B). As set forth at MPEP § 2107.02(II)(A), a disclosure that identifies a particular biological activity of a

compound and explains how that activity can be utilized in a particular therapeutic application of the compound does contain an assertion of specific and substantial utility. Here, the disclosure identifies a particular biological activity (binding oxidized low-density lipoproteins, internalizing them into endothelial cells, and destroying them), and explains how that activity can be utilized in a particular therapeutic application (treatment of atherosclerosis).

In entering the § 101 rejection, the Office Action (at page 6, second para.) directs Applicants' attention to the prior § 101 rejection set forth in the October 25, 2005 Office Action. The prior § 101 rejection notes that sequence alignments and percent similarities are not disclosed. However, Applicants respectfully submit that this has no bearing on whether a specific and substantial utility has been asserted.

B. The asserted utility is credible.

In most cases, an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement. See MPEP § 2107(III)(A). To overcome this presumption, Office personnel must establish that it is more likely than not that a person of ordinary skill would not believe the assertion. See *id.* Here, Applicants respectfully submit that the Office Action does not make such a showing. Nevertheless, Applicants are providing further explanation as to why the person of ordinary skill would find the asserted utility to be credible.

As set forth at MPEP § 2107.03(II), evidence of structural similarity to a known compound combined with evidence of shared pharmacological activity with the known compound is supportive of an assertion of utility.

In the instant case, the specification refers to the known protein LOX-1, and to its role in the pathogenesis of atherosclerosis. See page 178, lines 28 to 31 of the specification. Applicants respectfully submit that the skilled artisan would have recognized that both LOX1 and the bn97-1 clone are members of the C-type lectin family, which has the structural characteristic of six cysteine residues conserved in the C-terminal. In the article *Nature*, vol. 386, 1997, pp. 73 to 77 (copy attached as Exhibit), which is cited at page 178, line 28 of the specification, it was determined on the basis of a sequence alignment between LOX1 and NKR-P1 that LOX1 belongs to the C-type lectin family. See *Nature*, Fig. 4b. Thus, Applicants respectfully submit that one skilled in the art would have also determined on the basis of sequence similarity that clone bn97_1 belongs to the C-type lectin family, and as such, has C-type lectin activity.

Further, Applicants respectfully submit that clone bn97_1 is now known as CLEC-1. As shown in the sequence alignment included with the April 20, 2006 Amendment, clone bn97_1 and CLEC-1 have identical coding sequences.

The article *Eur. J. Immunol.*, vol. 30, 2000, pp. 697 to 704 (copy attached to April 20, 2006 Amendment) describes that CLEC-1 (clone bn97_1) belongs to the C-type lectin family. CLEC-1 conserves the six cysteine residues that are typical of C-type lectins. See *Eur. J. Immunol.*, Figure 2(A). In addition, *Eur. J. Immunol.* describes that CLEC-1 (clone bn97_1) may bind lipoproteins, such as oxidized LDL, and may have a function as a scavenger receptor. See *Eur. J. Immunol.*, para. bridging pp. 700 and 701. Furthermore, this article discloses that CLEC-1 (clone bn97_1) is expressed in dendritic cells, which are known to be important in antigen capture, phagocytosis of apoptotic

bodies, and lipoprotein metabolism, and which relate to pathogenesis of atherosclerosis. See *id.*

In this regard, while *Eur. J. Immunol.* was published after the filing date of parent Provisional Application No. 60/093,045, it is noted that a post-filing reference can be used to show credibility of an asserted utility. See *In re Brana*, 34 USPQ2d 1436, 1444 n.19 (Fed. Cir. 1995) ("The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification....It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)."). A copy of this case was provided with the April 20, 2006 Amendment.

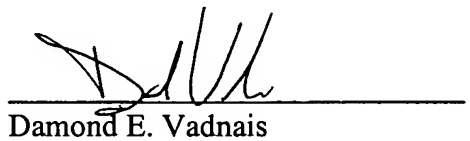
Conclusion

In view of the foregoing, it is respectfully submitted that the specification sets forth a specific and substantial utility. It is further respectfully submitted that this utility would be credible to one skilled in the art, as evidenced by the *Nature* and *Eur. J. Immunol.* documents. Accordingly, reconsideration and withdrawal of the § 101 rejection and related § 112 rejection are respectfully requested.

The application is believed to be in condition for allowance, and a Notice of Allowance is respectfully requested.

Applicants' undersigned attorney may be reached in our Costa Mesa, California, office by telephone at (714) 540-8700. All correspondence should be directed to our address given below.

Respectfully submitted,



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solution (rabbit; Sigma A-1420 supplemented with 22.6 mM Na_2HCO_3, 4.4 mM D-glucose, pH 7.4; maintained at 37 °C during recording). A piece of retina 2–4 mm on a side was placed onto a flat multi-electrode array for extracellular recording from the ganglion cell layer[10,25]. Results are reported from 71 cells in 4 salamander retinae, 55 cells in 2 rabbit retinae (Figs 1, 2, 3; Table 1), and 33 cells in 2 salamander retinae (Fig. 4). In the salamander, 14% of recorded ganglion cells were 'on' and 86% 'off'. In the rabbit, 33% were 'on', 51% 'off', and 16% had mixed response properties.

Stimulation. Stimuli were projected from a computer monitor onto a 3.25-mm-diameter aperture on the retina. Patterns consisted of a single uniform field of flickering light (Figs 1–3) or a flickering checkerboard with independently modulated square fields (Fig. 4), ranging in size, D, from 0.068 to 0.816 mm. The light intensity of each field was chosen every $\Delta t = 30$ ms at random from a gaussian probability distribution with mean M and standard deviation W. Contrast, C, was defined as W/M. The field size D sets the spatial scale of the stimulus. All experiments used white light[26] with a mean intensity of $M = 4.2\,\mathrm{mW\,m^{-2}}$ at the retina. The equivalent photon flux at the peak absorption wavelength (λ_{max}) was 5,680 photons $\mu m^{-2} s^{-1}$ for the salamander's red cone photoreceptor ($\lambda_{max} = 630$ nm), and 5,150 photons $\mu m^{-2} s^{-1}$ for the rabbit's green cone ($\lambda_{max} = 523$ nm)[27,28].

Analysis. In a typical experiment, we interleaved segments of two stimulus types in the order $A_1B_1A_2B_2A_3B_3$..., where the A_i represents segments with the same contrast and spatial scale, but different random flicker sequences, and B_i represent segments with another contrast or spatial scale. Individual segments of A and B lasted either 50 s or 100 s, and recordings extended over 25 to 100 AB trials. The mean firing rate at a given time around the A-to-B transition (Figs 1, 2, 4) was computed by counting the spikes in the corresponding short time bin of all the AB trials, and dividing by the number of trials and the length of the time bin. The first-order Wiener kernel (Fig. 3) of the response was computed by correlating the stimulus intensity $I(t)$ and the firing rate $R(t)$: $k(t) = \frac{1}{WC}\frac{1}{T}\int_0^T (I(t') - M)R(t' + t)dt'$. This represents the linear effect on the firing rate from a flash of light with integrated intensity $M{\cdot}\Delta t$. The kernel at a given time around the transition was computed by performing the integral only over the corresponding time bins of all individual trials. The spatial receptive field (Fig. 4) was determined from steady-state responses to checkerboard stimulation with 136-μm squares: the kernel $k(t)$ was computed for each square, and its peak amplitude plotted as a function of position. All error bars in figures represent the standard error across trials.

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1. Rushton, W. A. Visual Adaptation (The Ferrier Lecture, 1962). *Proc. R. Soc. Lond. B* 162, 20–46 (1965).
2. Barlow, H. B. Critical limiting factors in the design of the eye and visual cortex (The Ferrier Lecture, 1980). *Proc. R. Soc. Lond. B* 212, 1–34 (1981).
3. Shapley, R. & Enroth-Cugell, C. Visual Adaptation and Retinal Gain Controls. *Progr. Ret. Res.* 3, 263–346 (1984).
4. Laughlin, S. A simple coding procedure enhances a neuron's information capacity. *Z. Naturforsch.* 36, 910–912 (1981).
5. de Ruyter van Steveninck, R. R., Bialek, W., Potters, M. & Carlson, R. H. Statistical adaptation and optimal estimation in movement computation by the blowfly visual system. *Proc. IEEE Int. Conf. Systems, Man, and Cybernetics* 302–308 (1994).
6. DeWeese, M. & Bialek, W. Information flow in sensory neurons. *Il Nuovo Cimento* 17D, 733–738 (1995).
7. Blakemore, C. & Campbell, F. W. On the existence of neurones in the human visual system selectively sensitive to the orientation and size of retinal images. *J. Physiol.* 203, 237–260 (1969).
8. Ohzawa, I., Sclar, G. & Freeman, R. D. Contrast gain control in the cat's visual system. *J. Neurophysiol.* 54, 651–667 (1985).
9. Sakai, H. M. & Naka, K. Signal transmission in the catfish retina. V. Sensitivity and circuit. *J. Neurophysiol.* 58, 1329–1350 (1987).
10. Meister, M., Pine, J. & Baylor, D. A. Multi-neuronal signals from the retina: acquisition and analysis. *J. Neurosci. Meth.* 51, 95–106 (1994).
11. Shapley, R. M. & Victor, J. D. The effect of contrast on the transfer properties of cat retinal ganglion cells. *J. Physiol.* 285, 275–298 (1978).
12. Victor, J. D. The dynamics of the cat retinal X cell centre. *J. Physiol.* 386, 219–246 (1987).
13. Wang, J.-L. & Naka, K.-I. Contrast gain control in the lower vertebrates retinas. *Soc. Neurosci. Abstr.* 21, 1644 (1995).
14. Copenhagen, D. R. & Green, D. G. The absence of spread of adaptation between rod photoreceptors in turtle retina. *J. Physiol.* 369, 161–181 (1985).
15. Dacheux, R. F. & Raviola, E. Horizontal cells in the retina of the rabbit. *J. Neurosci.* 2, 1486–1493 (1982).
16. Hare, W. A. & Owen, W. G. Spatial organization of the bipolar cell's receptive field in the retina of the tiger salamander. *J. Physiol.* 421, 223–245 (1990).
17. Werblin, F., Maguire, G., Lukasiewicz, P., Eliasof, S. & Wu, S. M. Neural interactions mediating the detection of motion in the retina of the tiger salamander. *Visual Neurosci.* 1, 317–329 (1988).
18. Bloomfield, S. A. Relationship between receptive and dendritic field size of amacrine cells in the rabbit retina. *J. Neurophysiol.* 68, 711–725 (1992).
19. Barlow, H. B. & Hill, R. M. Evidence for a physiological explanation of the waterfall phenomenon and figural after-effects. *Nature* 200, 1345–1347 (1963).
20. Schieting, S. & Spillmann, L. Flicker adaptation in the peripheral retina. *Vision Res.* 27, 277–284 (1987).
21. Albrecht, D. G., Farrar, S. B. & Hamilton, D. B. Spatial contrast adaptation characteristics of neurones recorded in the cat's visual cortex. *J. Physiol.* 347, 713–739 (1984).
22. Ho, W. A. & Berkley, M. A. Evoked potential estimates of the time course of adaptation and recovery to counterphase gratings. *Vision Res.* 28, 1287–1296 (1988).
23. Giaschi, D., Douglas, R., Marlin, S. & Cynader, M. The time course of direction-selective adaptation in simple and complex cells in cat striate cortex. *J. Neurophysiol.* 70, 2024–2034 (1993).
24. Barlow, H. B. & Bridley, G. S. Inter-ocular transfer of movement aftereffects during pressure binding of the stimulated eye. *Nature* 200, 1347 (1963).
25. Meister, M., Lagnado, L. & Baylor, D. A. Concerted signaling by retinal ganglion cells. *Science* 270, 1207–1210 (1995).
26. Brainard, D. Calibration of a computer controlled color monitor. *Color Res. Appl.* 14, 23–34 (1989).
27. Dawis, S. M. Polynomial expressions of pigment nomograms. *Vision Res.* 21, 1427–1430 (1981).
28. Nuboer, J. F. W. & Moed, P. J. Increment-threshold spectral sensitivity in the rabbit. *J. Comp. Physiol.* 151, 353–358 (1983).

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An endothelial receptor for oxidized low-density lipoprotein

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Endothelial dysfunction or activation elicited by oxidatively modified low-density lipoprotein (Ox-LDL) has been implicated in the pathogenesis of atherosclerosis[1–4], characterized by intimal thickening and lipid deposition in the arteries. Ox-LDL and its lipid constituents impair endothelial production of nitric oxide, and induce the endothelial expression of leukocyte adhesion molecules and smooth-muscle growth factors, which may be involved in atherogenesis[5–7]. Vascular endothelial cells in culture[8,9] and *in vivo*[10,11] internalize and degrade Ox-LDL through a putative receptor-mediated pathway that does not involve macrophage scavenger receptors[12–15]. Here we report the molecular cloning, using expression cloning strategy, of an Ox-LDL receptor from vascular endothelial cells. The cloned receptor is a membrane protein that belongs structurally to the C-type lectin family, and is expressed *in vivo* in vascular endothelium and vascular-rich organs.

A cDNA library of cultured bovine aortic endothelial cells (BAEC), which bind, internalize and degrade Ox-LDL, was used for expression cloning. COS-7 cells transfected with a single clone, pBLOX-1, exhibited prominent uptake of DiI-labelled Ox-LDL. To characterize the protein encoded by pBLOX-1, designated lectin-like Ox-LDL receptor (LOX-1), as a receptor for Ox-LDL, we have transfected pBLOX-1 into CHO-K1 cells and established a cell line stably expressing bovine LOX-1 (BLOX-1-CHO). An antiserum was also developed using bacterially expressed extracellular domain of LOX-1(61–270) as an antigen. Immunoblotting of total cell lysate of BLOX-1-CHO, using anti-LOX-1 antiserum, showed a single band with approximate relative molecular mass 50,000 (M_r 50K). This particular band was not detected in cell lysate from untransfected CHO-K1 cells (Fig. 1a). A band with 50K on the same position of the

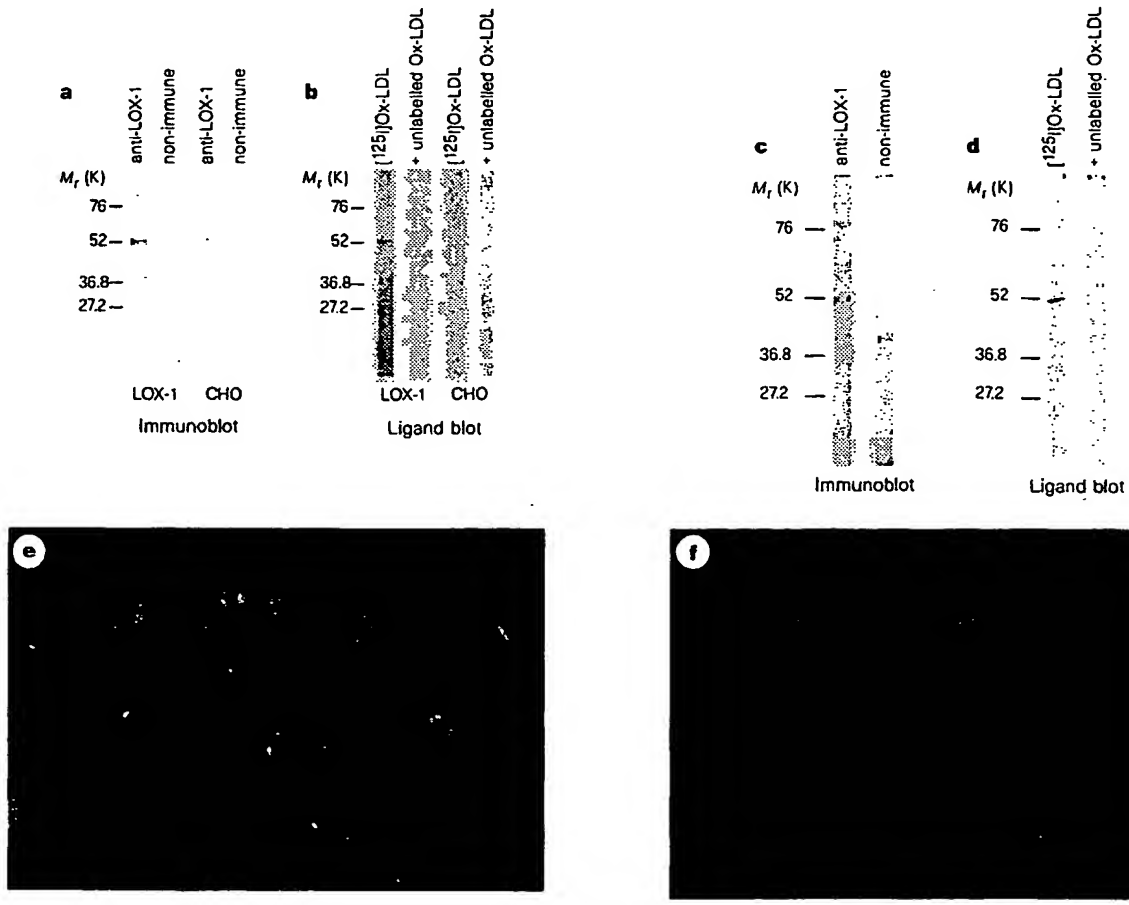

Figure 1 LOX-1 is a protein of M_r 50K that is expressed on the cell surface of bovine aortic endothelial cells. **a**, Immunoblotting of BLOX-1-CHO and untransfected CHO-K1 with anti-LOX-1 antiserum. A band at 50K was detected in BLOX-1-CHO, but not in untransfected CHO-K1. **b**, Ligand blotting of BLOX-1-CHO and untransfected CHO-K1 cells using [^{125}I]Ox-LDL as a ligand. A similar band of ~50K was detected in BLOX-1-CHO but not in untransfected CHO-K1. Addition of a 100-fold excess of unlabelled Ox-LDL abolished the binding of [^{125}I]Ox-LDL. **c**, Immunoblotting of BAEC by anti-LOX-1 antiserum. A band of 50K was detected in BAEC. A monoclonal antibody for bovine LOX-1 also detected the 50K band (data not shown). **d**, Ligand blotting of BAEC using [^{125}I]Ox-LDL as a ligand. A 50K band was detected as described in immunoblotting of BAEC, as well as both immunoblotting and ligand blotting of BLOX-1-CHO. **e**, **f**, Indirect immunofluorescence of non-permeabilized BLOX-1-CHO (**e**) and BAEC (**f**) with anti-bovine LOX-1 antiserum. Cells were fixed with 3% paraformaldehyde, and subsequently incubated with anti-LOX-1 antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. Immunofluorescence microscopy shows that LOX-1 is expressed on the cell surface. Signals from the intracellular space of BLOX-1-CHO are leakage of the fluorescence of DiI-Ox-LDL incubated before cell fixation. Staining with preimmune serum did not show any significant signals (data not shown).

same membrane was detected by ligand blotting, using [^{125}I]-labelled Ox-LDL as a ligand (Fig. 1b). Both immunoblot and ligand blot of BAEC proteins similarly stained a single band on the same position of the same membrane, suggesting that this 50K protein binds Ox-LDL in BAEC (Fig. 1c, d). Indirect immunofluorescence of non-permeabilized BLOX-1-CHO cells showed that anti-LOX-1 antiserum bound to the cell surface of BLOX-1-CHO (Fig. 1e) and BAEC (Fig. 1f), but not to untransfected CHO-K1 cells (data not shown), demonstrating that LOX-1 is expressed on the cell surface.

To further confirm the function of LOX-1 as a receptor for Ox-LDL, binding and proteolytic degradation of [^{125}I]-labelled Ox-LDL, as well as internalization of DiI-labelled Ox-LDL, were examined. Binding of [^{125}I]-labelled Ox-LDL to both BLOX-1-CHO and BAEC were effectively blocked by 100-fold excess amounts of unlabelled Ox-LDL. The amounts of [^{125}I]-labelled Ox-LDL binding to BLOX-1-CHO cells were no less than those to CHO cells expressing bovine type II macrophage scavenger receptor (281 and 110 ng mg^{-1} cellular protein, respectively)[13]. Two independent monoclonal antibodies directed to bovine LOX-1 significantly inhibited [^{125}I]-labelled Ox-LDL binding to both BLOX-1-CHO and BAEC (Fig. 2). Fluorescence microscopy of BLOX-1-CHO incubated with DiI-labelled Ox-LDL showed that BLOX-1-CHO cells, but not untransfected CHO-K1 cells, internalized significant amounts of DiI-labelled Ox-LDL (Fig. 3a), which was also blocked by excess amounts of unlabelled Ox-LDL but not by unlabelled native LDL (data not shown). [^{125}I]-labelled Ox-LDL internalized into BLOX-1-CHO can be proteolytically degraded, as previously reported in cultured endothelial cells[9]. This degradation was effectively suppressed by excess amounts of unlabelled Ox-LDL but not by unlabelled native LDL (Fig. 3c). Polyinosinic acid, which inhibits endothelial binding of Ox-LDL[11], also significantly blocked binding of [^{125}I]-labelled Ox-LDL in BLOX-CHO. Thus LOX-1 seems to be expressed on the cell surface on BAEC, and can support binding, internalization and proteolytic degradation of Ox-LDL.

We screened a cDNA library constructed from human lung, and subsequently cloned cDNA encoding the human homologue of LOX-1. A cell line stably expressing human LOX-1 (HLOX-1-CHO) also exhibited the activity of the uptake of DiI-labelled Ox-LDL (Fig. 3b), which was displaced by unlabelled Ox-LDL but not by native LDL (data not shown). Degradation of [^{125}I]-labelled Ox-LDL by HLOX-1-CHO was also confirmed, and was inhibited by

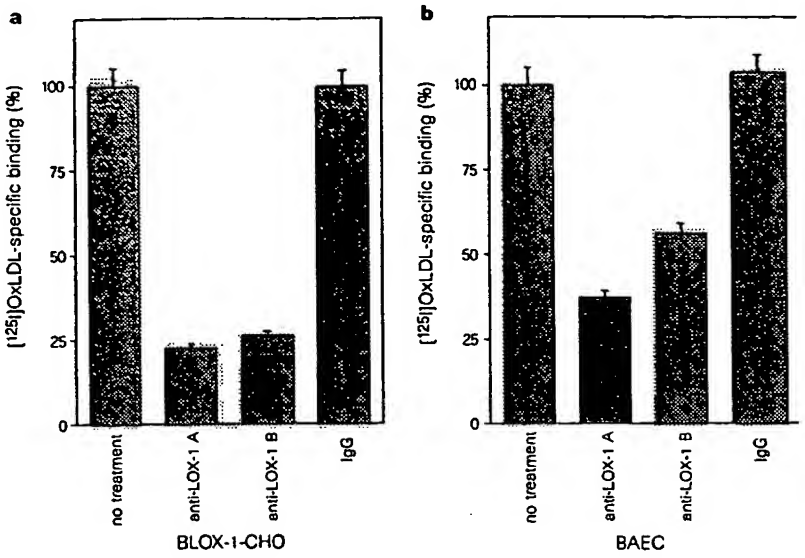

Figure 2 Binding of [^{125}I]Ox-LDL to BLOX-1-CHO (**a**) and BAEC (**b**). Cells were incubated with 5 μg ml^{-1} of [^{125}I]Ox-LDL on ice for 1 h, washed 3 times, and cell-bound radioactivities were measured by a gamma counter. Specific binding of Ox-LDL was calculated as displacable binding with 100-fold excess of (500 μg ml^{-1}) unlabelled Ox-LDL. Two independent anti-BLOX-1 monoclonal antibodies (30 μg ml^{-1}) or a comparable amount of non-immune mouse IgG was also included in incubation media. Addition of monoclonal antibodies directed to LOX-1, but not control non-immune IgG, significantly reduced the specific binding of [^{125}I]Ox-LDL to BAEC. The absolute values of specific binding of [^{125}I]Ox-LDL to BLOX-1-CHO and BAEC were 281 ± 7.8 per ng mg protein and 20 ± 1.2 ng per mg protein (mean ± s.e.m.), respectively.

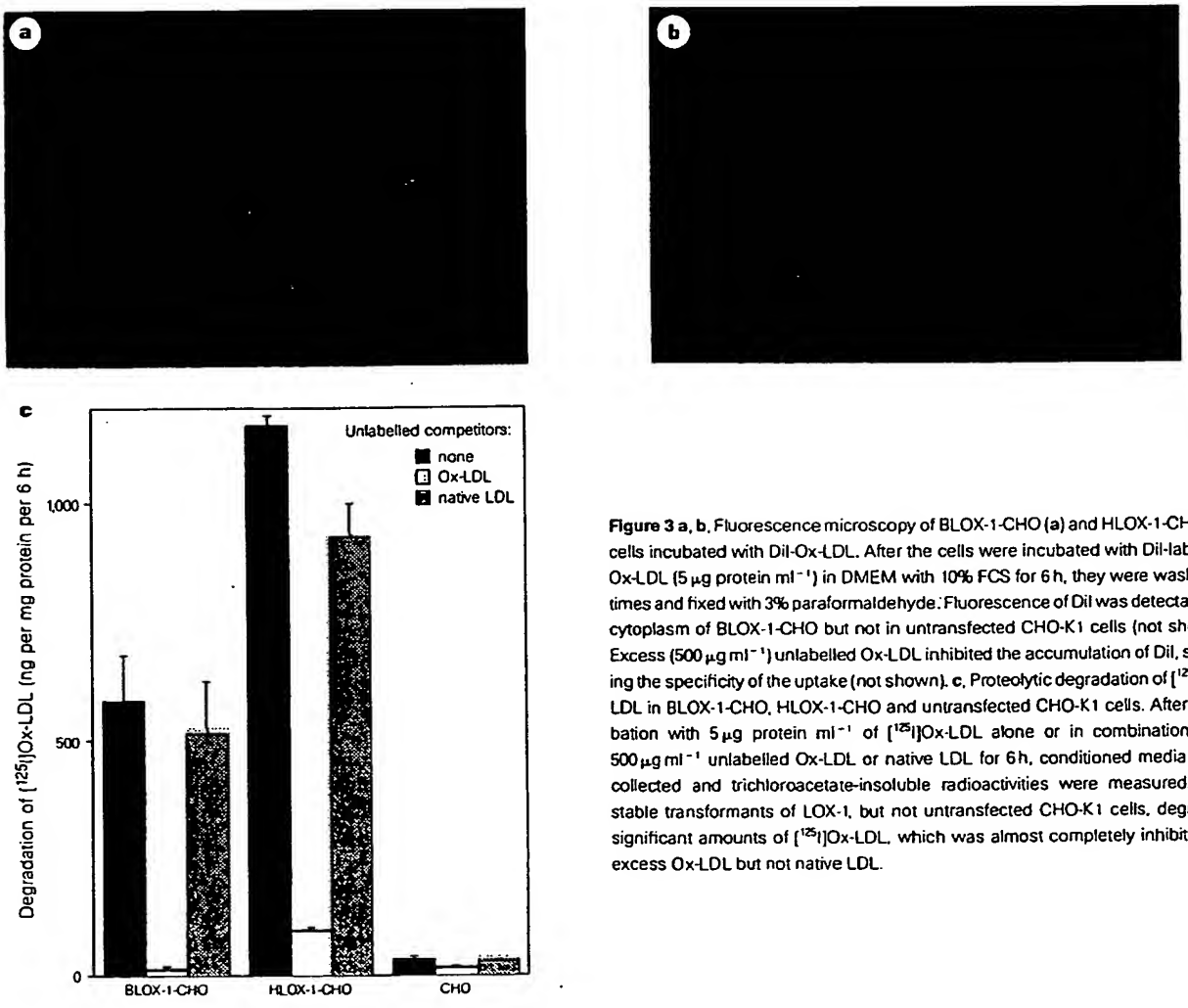

Figure 3 a, b, Fluorescence microscopy of BLOX-1-CHO (**a**) and HLOX-1-CHO (**b**) cells incubated with DiI-Ox-LDL. After the cells were incubated with DiI-labelled Ox-LDL (5 μg protein ml^{-1}) in DMEM with 10% FCS for 6 h, they were washed 3 times and fixed with 3% paraformaldehyde. Fluorescence of DiI was detectable in cytoplasm of BLOX-1-CHO but not in untransfected CHO-K1 cells (not shown). Excess (500 μg ml^{-1}) unlabelled Ox-LDL inhibited the accumulation of DiI, showing the specificity of the uptake (not shown). **c**, Proteolytic degradation of [^{125}I]Ox-LDL in BLOX-1-CHO, HLOX-1-CHO and untransfected CHO-K1 cells. After incubation with 5 μg protein ml^{-1} of [^{125}I]Ox-LDL alone or in combination with 500 μg ml^{-1} unlabelled Ox-LDL or native LDL for 6 h, conditioned media were collected and trichloroacetate-insoluble radioactivities were measured. The stable transformants of LOX-1, but not untransfected CHO-K1 cells, degraded significant amounts of [^{125}I]Ox-LDL, which was almost completely inhibited by excess Ox-LDL but not native LDL.

unlabelled Ox-LDL but not by native LDL (Fig. 3c). These similar results with the human homologue of LOX-1 demonstrate that LOX-1 works as a receptor for Ox-LDL in both species.

The cDNA for bovine LOX-1 cloned from BAEC contained an open reading frame of 810 base pairs that encodes a protein of 270 amino-acid residues with a calculated M_r of 30,872. The expressed protein in CHO-K1 cells and BAEC had an M_r of 50K (Fig. 1a, c), which may result from glycosylation of four potential N-linked glycosylation sites located on C-terminal domain. In the amino-terminal cytoplasmic domain, several potential phosphorylation sites were found (Thr 21 and Ser 28 for protein kinase C; Thr 2 for casein kinase II), suggesting that phosphorylation of these sites may transmit biological signals or regulate the function of LOX-1. Human LOX-1 has 273 amino acids, and an M_r of 30,939. The structure of human LOX-1 was homologous to bovine LOX-1, with 72% identity in the amino-acid sequences. Homology was further conserved in the lectin domain (see below); 81% was identical in the amino-acid sequences (Fig. 4a).

LOX-1 has the structure conserved in the C-type lectin family[16]. It has a type II membrane protein structure with a short N-terminal hydrophilic and a long carboxy-terminal hydrophilic domain, separated by a hydrophobic domain of 26 amino acids. Six repeats of cysteines in the homologous lectin domain were also found. LOX-1 has the highest homology with NKR-P1 family proteins expressed on the surface of natural killer (NK) cells[17], which are involved in target-cell recognition and NK-cell activation (Fig. 4b).

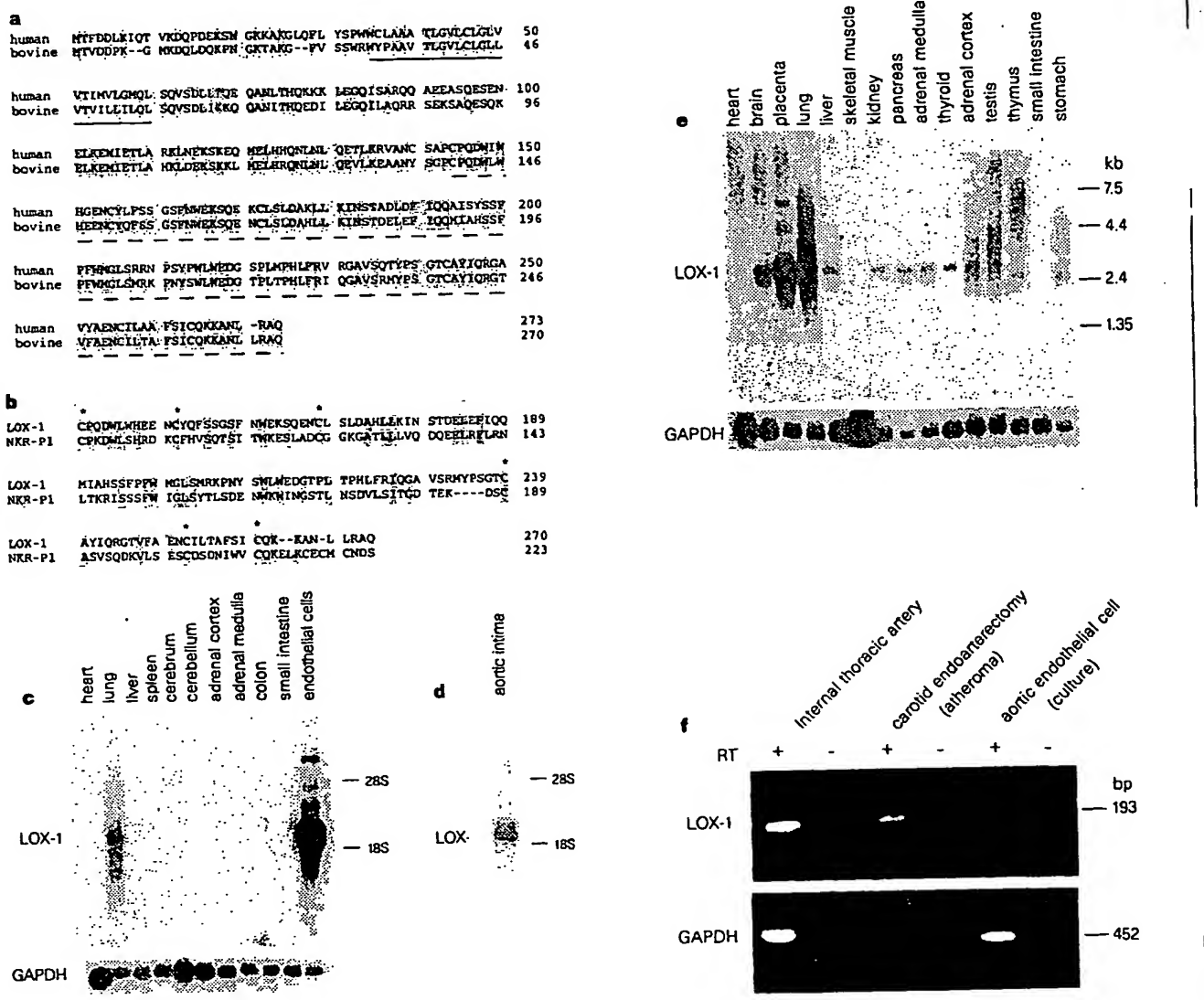

Figure 4 a, Comparison of amino-acid sequences between human and bovine LOX-1. Conserved amino-acid sequences in both species are shaded. The putative transmembrane domain is underlined, and the C-type lectin domain is indicated by dashed underline. **b**, Alignment of C-type lectin domains of LOX-1 and rat NKR-P1. The positions of six cysteine residues are conserved (asterisks). **c–e**, Northern blot analyses of bovine tissues and cultured aortic endothelial cell (**c**), bovine aortic intima (**d**), and human tissues (**e**). LOX-1 mRNA was detected as bands for 2.3 kb and 2.8 kb in bovine and human tissues, respectively. To control the amounts of mRNA loaded, blots were reprobed with cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Note that the exposure of bovine membrane (**c**) was shorter than that of human membrane (**e**) because of the high level of expression of LOX-1 mRNA in cultured bovine aortic endothelial cells. **f**, RT-PCR of human internal thoracic artery, a specimen from carotid endoarterectomy, and cultured human aortic endothelial cells. LOX-1 cDNA fragments with 193 base pairs were amplified by primer pair for human LOX-1 cDNA. To control the amounts of mRNA 452 base-pair fragments of GAPDH cDNA were amplified using Amplimer set (Clontech) from the same RNA preparations. These cDNA fragments were undetectable when reverse transcription was omitted.

LOX-1, like NKR-P1 might also transmit some biological signals and thereby activate endothelial cells after ligation to Ox-LDL. LOX-1 does not have any homology with known receptors for Ox-LDL which were mainly found in macrophages[13,14,18–21]. Therefore, molecular mechanisms of binding of this C-type lectin-like molecule to Ox-LDL may be distinct from those previously found for Ox-LDL receptors, and remain to be clarified. The C-type lectin family includes 'selectins' that are involved in cell-cell recognition, including endothelial-leukocyte adhesion. Macrophage class A scavenger receptors were involved in divalent cation-independent adhesion of macrophages to substrates[22]. LOX-1 may also, therefore, have additional (patho)physiological functions, including cell–cell and cell–matrix adhesion.

To define whether LOX-1 is expressed *in vivo*, as well as in cultured endothelial cells, northern blot analyses and the polymerase chain reaction coupled with reverse transcription (RT–PCR) were performed. Northern blot analyses of various tissues revealed that aortic intima and vascular-rich organs, such as placenta, lungs, brain and liver, express LOX-1 mRNA *in vivo* in physiological conditions (Fig. 4c–e). RT–PCR showed that human LOX-1 is expressed in normal thoracic and carotid arterial cells including atheromatous lesions, as well as cultured aortic endothelial cells (Fig. 4f). The 3′-non-coding region of bovine LOX-1 cDNA contains seven mRNA unstabilizing signals, AUUUA[23], indicating that mRNA for LOX-1 may have a short intracellular half-life. This suggests that expression of LOX-1 might be dynamically regulated by certain biological stimuli that are relevant to atherogenesis. Certain inflammatory cytokines, Ox-LDL and its atherogenic lipid constituent can upregulate the expression of LOX-1 (data not shown). Although the potential roles of LOX-1 in atherogenesis are not yet fully understood, Ox-LDL uptake through this receptor expressed on the surface of vascular endothelium may be involved in endothelial activation or dysfunction in atherogenesis. □

Methods

Preparation of Ox-LDL. Human plasma LDs (relative density 1.019–1.063) were isolated by sequential ultracentrifugation, and oxidative modification of LDL was performed with Cu^{2+} *in vitro*. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (around 10 nmol malondialdehyde equivalent per mg protein in Ox-LDL)[9]. Agarose gel electrophoresis showed increased electrophoretic mobility and minimal aggregation of Ox-LDL particles. LDL was labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)[24] (Molecular Probes).

cDNA cloning of bovine LOX-1. A cDNA library of bovine endothelial cells was constructed in a mammalian expression vector pME18S[25]. The plasmid library consisted of 7×10^5 independent clones. The library was once amplified and used for transfection.

For COS-7 transfection and cell sorting, 10 μg of plasmids were transfected into COS-7 cells by using Lipofectamine (Gibco). COS-7 cells were incubated 48 h after transfection with 3 μg protein per ml of DiI-labelled Ox-LDL for 12 h. DiI-positive COS-7 cells were sorted by a FACS Vantage cell sorter (Becton Dickinson). Episomal plasmids were collected from the sorted cells[26] and electroporated into DH10B. Plasmid DNAs were purified after amplification and subjected to another round of transfection and sorting. After three rounds of selection, plasmids from single clones were prepared and individually transfected into COS-7 cells.

Stable transformant of LOX-1. pBLOX-1 (1 μg) and pSV2bsr (10 ng) (Funakoshi, Japan) were co-transfected into CHO-K1 cells by the calcium-phosphate transfection method. Stable transformants were selected under the existence of 10 μg ml^{-1} of blasticidin S (Funakoshi).

Antibodies against LOX-1. A cDNA fragment covering the extracellular domain of bovine LOX-1 (amino acids 61–270) was amplified by PCR with a pair of primers tagged with a *Bam*HI restriction site. The amplified fragment was digested with *Bam*HI and subcloned into the *Bam*HI site of pQE10 vector (QIAGEN). Protein synthesis and purification of the extracellular domain of LOX-1 were performed using the QIAexpress system (QIAGEN). The protein was used as the antigen for immunizing mice. Hybridomas producing monoclonal antibodies were made by standard procedures and screened by enzyme-linked immunosorbent assay. Two independent clones that showed significant inhibition in the binding of [^{125}I]-Ox-LDL to BLOX-1-CHO were selected.

Immunoblot and ligand blot analyses. Cells were solubilized directly in the sample buffer for SDS-PAGE without reducing reagents. The extracts were separated by SDS-PAGE in non-reducing conditions and blotted onto nylon membranes. After blocking with Block Ace (Snow Brand, Japan), the membranes were incubated with 10 μg ml^{-1} [^{125}I]-Ox-LDL with or without 1 mg ml^{-1} unlabelled Ox-LDL in 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 90 mM NaCl, 25% (v/v) Block Ace at room temperature. The membranes were washed in the same buffer without ligands and autoradiographed with BAS2000 system (Fuji). Immunostaining of the membranes with an anti-bovine LOX-1 antibody was performed using biotinylated second antibody and peroxidase-conjugated avidin-biotin complex (Vector) and Immunostain kit (Konica).

cDNA cloning of human LOX-1. A cDNA library of human lung primed with random primer and oligo(dT) primer was screened by full-length bovine LOX-1 cDNA. A single positive clone (λhLOX-1) was used which covers human LOX-1(465–1192). A 5′-upstream region of human LOX-1 cDNA was obtained from poly(A)$^+$ RNA from human placenta by 5′-RACE using specific primer corresponding to the antisense strand of human LOX-1(555–581) (ref. 27). The cDNA sequence was confirmed by three independent clones. Sequence homology was analysed by Geneworks (Intelligenetics).

Northern blot analyses. Poly(A)$^+$ RNA (5 μg per lane), prepared from bovine tissues, was separated by a formaldehyde/1.1% agarose gel electrophoresis, and transferred to a nylon membrane (Gene Screen Plus, DuPont). These membranes and multiple tissue northern blot of human tissues (Clontech) were hybridized with cDNA of bovine and human LOX-1 labelled with [α-^{32}P]dCTP by random priming method, respectively. Hybridization was performed at 42 °C in 2 × SSC, 50% formamide, 1% SDS, 0.1 mg ml^{-1} salmon sperm DNA, 10% dextran sulphate. The membrane was washed in 0.2 × SSC/0.1% SDS at 65 °C, and autoradiographed with BAS2000 system (Fuji).

RT–PCR. Total RNA (500 ng) extracted from fragments of human arteries was reverse transcribed with random hexamer using Super Script (Gibco). As much as 5% of the reverse-transcribed materials were amplified with LA-Taq DNA polymerase (Takara) using a primer pair specific to human LOX-1 (sense primer, 5′-TTACTCTCCATGGTGGTGCC-3′, antisense primer, 5′-AGCTTCTTCTGCTTGTTGCC-3′). For PCR, 35 cycles were used at 94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min.

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1. Brown, M. S. & Goldstein, J. L. *Annu. Rev. Biochem.* 52, 223–261 (1983).
2. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witztum, J. L. *N. Engl J. Med.* 320, 915–924 (1989).
3. Kita, T. *et al. Proc. Natl Acad. Sci. USA* 84, 5928–5931 (1987).
4. Ross, R. *Nature* 368, 801–809 (1993).
5. Gimbrone, M. A. J., Cybulsky, M. I., Kume, N., Collins, T. & Resnick, N. *Ann. NY Acad. Sci.* 748, 122–132 (1995).
6. Kume, N. & Gimbrone, M. A. J. *J. Clin. Invest.* 93, 907–911 (1994).
7. Kugiyama, K., Kerns, S. A., Morrisett, J. D., Roberts, R. & Henry, P. D. *Nature* 344, 160–162 (1990).
8. Stein, O. & Stein, Y. *Biochim. Biophys. Acta* 620, 631–635 (1980).
9. Kume, N., Arai, H., Kawai, C. & Kita, T. *Biochim. Biophys. Acta* 1091, 63–67 (1991).
10. Pitas, R. E., Boyles, J., Mahley, R. W. & Bissel, D. M. *J. Cell. Biol.* 100, 103–117 (1985).
11. van Berkel, T. J. C., De Rijke, Y. B. & Kruijt, J. K. *J. Biol. Chem.* 266, 2282–2289 (1991).
12. Krieger, M. *et al. J. Biol. Chem.* 268, 4569–4572 (1993).
13. Kodama, T. *et al. Nature* 343, 531–535 (1990).
14. Rohrer, L., Freeman, M., Kodama, T., Penman, M. & Krieger, M. *Nature* 343, 570–572 (1990).
15. Bickel, P. E. & Freeman, M. W. *J. Clin. Invest.* 90, 1450–1457 (1992).
16. Drickamer, K. J. *J. Biol. Chem.* 263, 9557–9560 (1988).
17. Giorda, R. *et al. Science* 249, 1298–1300 (1990).
18. Endemann, G. *et al. J. Biol. Chem.* 268, 11811–11816 (1993).
19. Acton, S. L., Scherer, P. E., Lodish, H. F. & Krieger, M. *J. Biol. Chem.* 269, 21003–21009 (1994).
20. Standon, L. W., White, R. T., Bryant, C. M., Protter, A. A. & Endemann, G. *J. Biol. Chem.* 267, 22446–22451 (1992).
21. Ramprasad, M. P. *et al. Proc. Natl Acad. Sci. USA* 92, 9580–9584 (1995).
22. Fraser, I., Hughes, D. & Gordon, S. *Nature* 364, 343–346 (1993).
23. Show, G. & Kamen, R. *Cell* 46, 659–667 (1986).
24. Stephan, Z. F. & Yurachek, E. C. *J. Lipid Res.* 34, 325–330 (1993).
25. Ikura, T. *et al. Biochem. Biophys. Res. Commun.* 203, 1417–1422 (1994).
26. Hirt, B. *J. Mol. Biol.* 26, 365–369 (1967).
27. Frohman, M. A., Dush, M. K. & Martin, G. R. *Proc. Natl Acad. Sci. USA* 85, 8998–9002 (1988).

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